CARBOHYDRATE LEVELS IN RAT TISSUES-
EFFECTS OF DIETARY CREATINE AND STUDIES OF
GLUCOSE-C^{14} INCORPORATION.

by

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I N T R O D U C T I O N

It has been approximately eighty years since creatine was first isolated from meat extract, and yet, the role of this simple, nonessential amino acid in the integrated functioning of mammalian metabolism remains unclear. It is ironical that the most recent comprehensive monograph (2) on creatine metabolism, appearing in 1943, failed to appreciate to the fullest extent the findings of Borsook et al. (7) and Bloch et al. (4,5). These workers, utilizing some of the early applications of isotope methodology to research problems, established the biosynthetic pathway of creatine and its irreversible conversion to creatinine as it is accepted today. Equally as important, they established the supremacy of radiobiochemistry as a research technique over the previous crude approach of balance studies with creatine and creatinine which had led Beard to conclude erroneously that the predominant pathway was the conversion of creatinine to creatine.

Once the pathways of synthesis and degradation of creatine were established, interest in creatine metabolism decreased. Bloch et al. (6) excluded the occurrence of any major catabolic pathway other than that of creatinine formation, followed by excretion of this compound; however, they were careful to point out that their studies

did not exclude the possibility that extensive creatine degradation by other routes may occur when the compound is administered in large quantities. It was only recently that interest has been revived in this possibility, mainly by the observations that the ingestion of diets supplemented with creatine by the chick (42) and the rat (16,39,40) produces large decreases in vitro of the enzyme, transamidinase, in the liver and the kidney, respectively.

It has been suggested that the activity of this enzyme serves as a controlling mechanism for creatine biosynthesis (16,44); however, other investigators have failed to find any decrease in total body creatine or creatine phosphate in protein-depleted rats, despite an 85% decrease in kidney transamidinase by in vitro assay (38). Similarly, the failure of prior creatine feeding or abnormally high creatine blood levels to influence the ability of the isolated perfused rat liver to synthesize creatine, has cast doubt as to the importance of low transamidinase levels (17). It was noted by some investigators that the apparent creatine inhibition of transamidinase could be readily reversed in mice and rats by feeding glycine (12,39). Recently Fitch (14) has pointed out that this reversal of transamidinase inhibition is markedly dependent on the basic composition of the diet which is fed and to which

creatine is added, and has suggested that perhaps it is a particular balance of amino acids that is required for maximal enzyme activity, rather than the ability of any single amino acid to inhibit or stimulate the level of this enzyme. In a recent paper it was shown that the injection of large amounts of creatine intraperitoneally into normal rats failed to reveal any significant amount of destruction of the compound, since 85% of the creatine was recovered unchanged in the urine, and 6% was distributed in the body (41). In the light of the above findings, it is apparent that much remains to be established regarding the significance of dietary creatine.

Some of the known relations of creatine phosphate and carbohydrate metabolism are of interest. In this regard it has long been recognized that in basic disturbances of carbohydrate metabolism, as in diabetes mellitus, creatinuria occurs. Similarly, carbohydrate starvation results in creatinuria which can be abolished by feeding sugar or protein. The dependence of creatine phosphate formation on oxidative phosphorylation resulting from glycolysis and the aerobic oxidation of pyruvate and lactate is further evidence of the close interactions that exist. Ord and Stocken (25) using blood perfusion studies with the hind limb of the rat have demonstrated that rat skeletal muscle loses creatine reversibly with a fall in circulating glucose from 150 to 50 mg per 100 ml of blood. They also have shown that

compounds known to interfere with glucose utilization, such as sodium iodoacetate and dinitrophenol, produce a loss of creatine from muscle.

In addition to the observation that alterations of carbohydrate metabolism can affect creatine levels, there is evidence to suggest that the reverse may be true. In 1929, Hill et al. (20) reported that creatine given subcutaneously or by mouth to fasting dogs decreased the blood sugar level, although never to a point which produced convulsions. The administration of creatine with glucose in an oral glucose tolerance test decreased or abolished the subsequent rise in blood sugar. Equivalent doses of creatine on a weight basis administered to fasting rabbits failed to produce hypoglycemia (26). It was reported that a group of German workers found a hypoglycemic effect in humans after the intravenous administration of 2 g of creatine, an effect comparable to that resulting from 10 units of insulin (2). The effect, however, was not additive to that of insulin.

Studies of effects on carbohydrate metabolism of dietary creatine in rats have been reported. Todd and Allen (35) had observed that rats prefed a diet containing 10% glycine exhibited marked hepatic glycogenesis in a 3-hour recovery period following a stress of swimming in cold water. More recently it was observed that the redeposition of liver glycogen was eliminated and blood

glucose levels were decreased during the recovery period in rats prefed the same diet with 1% creatine added (36). In as much as the glycogenic action of glycine has been attributed, at least in part, to a stimulation of gluconeogenesis, it was postulated that this effect of dietary creatine was to inhibit the increased gluconeogenesis resulting from dietary glycine and stress. Dietary creatine was observed to decrease liver glycogen in the resting state also, but no changes were found in blood glucose levels at this time. No changes were found in muscle glycogen at either point in the experiment for animals receiving the creatine-supplemented ration.

Since the previous observations indicate that dietary creatine may significantly influence carbohydrate metabolism, the experiments to be described were designed to study this relation by a more rigid approach. The experiments were planned to reduce carbohydrate stores in rats to a basal level by fasting, and then, to standardize feeding conditions for all animals by allowing a limited diet intake during a restricted period of time. After the rations containing either 10% glycine or 10% glycine plus 10% creatine were eaten, the levels of blood glucose and tissue glycogen would be examined for changes at various time intervals in both groups. With this experimental design, the onset,

magnitude, and duration of any effects of dietary creatine might be more closely determined and serve to help indicate the underlying mechanism. To study the possibility that the effects on carbohydrate metabolism might be mediated by an altered rate of gluconeogenesis, preliminary studies of the pattern of glucose-C^{14} incorporation into blood glucose and tissue glycogen were carried out.

MATERIALS AND METHODS

Animals:

Male, Sprague-Dawley strain rats weighing from 220 to 260 g were removed from Purina Laboratory Chow stock diet and fasted for 48 hours prior to the experiment, at which time they weighed from 180 to 220 g.

Diet and Feeding:

Water was allowed at all times. Following the 48-hour fast the animals were weighed, and paired on this basis. The glycine-fed animals were given 2.0 g of a synthetic diet, as previously described by Todd and Talman (37). The diet has the following percentage composition by weight: casein, 16; Wesson salt mix, 5; Brewer's yeast, 10; cod liver oil, 2; Wesson Oil, 5; white corn dextrin, 44; glycine, 10; and dextrose, 8. The creatine-fed animals received 2.0 g of the same diet containing an additional 200 mg of creatine.

All animals were allowed 30 minutes in which to consume the 2.0 g of diet. Thirty minutes from the time the food can had been placed in the cage, it was removed, and animals leaving more than 10 mg of diet uneaten were discarded from the experiment. Occassionally animals were noted to tarry in beginning to eat. These animals were allowed an additional 10 minutes to complete their meal, and their time schedule was delayed accordingly.

Tissue Preparation:

At various intervals from 2 to 16 hours following removal of the food can from the cage, animals from both groups were sacrificed by injecting 10 mg of pentobarbital sodium intraperitoneally 5 minutes prior to the time of sacrifice. With the animal well anesthetised, an abdominal slit was made and carried into the left portion of the thorax through the left hemi-diaphragm. Two ml of blood was obtained by direct cardiac puncture with a heparinized syringe and was laked in water for determination of blood glucose. The total liver was quickly dissected free, blotted briefly on a paper towel to remove excess blood, and minced by one pass through a custom-built garlic press. Within sixty seconds of the time the abdomen was entered, a sample of minced liver for isolation of glycogen was added to a tared centrifuge tube containing 30% KOH. The whole right gastrocnemius muscle was removed and added to another tared centrifuge tube containing 30% KOH for isolation of muscle glycogen.

Both tissue glycogen and blood glucose were determined as glucose by the enzymatic method of Salomon and Johnson (28). All determinations were incubated for 1 hour at room temperature and read in a Beckman model DU spectrophotometer at 365 mu. This method gave consistently reproducible standard curves, and tissue

determinations averaged 95% of the values obtained on the same samples by the Somogyi modification of the Shaffer-Hartman method (30).

The laked blood was deproteinized with NaOH and $ZnSO_4$ according to the method of Somogyi (29). Aliquots of the filtrate were assayed for glucose.

Glycogen was isolated from liver and muscle as described by Good et al. (18), modified by introducing one alcohol reprecipitation of the glycogen. The glycogen was hydrolyzed for 2.5 hours at $100^{\circ}C$ in 5 ml of N H_2SO_4. The hydrolysates were nearly neutralized with N NaOH and diluted to a volume that provided a concentration range of glucose between 7.5 and 200 ug per ml. Aliquots of these hydrolysates were assayed for glucose, and calculated to glycogen by correcting for the additional weight of glucose derived from the water addition during hydrolysis.

Isotope Studies:

In the studies involving carbon-14, the diets and experimental procedures were exactly as described for the nonisotopic experiments, except that on the fifth hour after the meal, each animal received 11 uC of uniformly labeled glucose-C^{14} (New England Nuclear Corp.- specific activity of 86 uC per mg glucose) in 0.5 ml volume of water injected intraperitoneally. Animals were sacrificed at hourly intervals for 5 hours following the injection of the tracer glucose. Determinations of blood glucose, liver glycogen, and muscle

glycogen were performed as previously described, except that a larger portion of minced liver was required for isolation of hepatic glycogen. In addition, the amount of glucose-C^{14} in tissue glycogen and blood glucose at hourly intervals during the 5-hour period was ascertained. Only the additional techniques required to prepare the material for radioassay will be described in this section.

After removing an aliquot of the deproteinized blood filtrate for glucose determination, the remainder was passed through a well-washed 0.6 X 10 cm ion exchange column containing Amberlite MB-3 resin to remove charged molecules, as described by Nadkarni et al. (23). This procedure was demonstrated in our laboratory to remove quantitatively amino acids, inorganic ions, and radioactive, charged substances other than glucose from the solutions. The columns were rinsed with water, and 50 ml of eluate was collected from each column. Since the glucose concentration and specific activity of the glucose were often too low for assay at this point, the solutions were concentrated by heating in an evaporating dish to 80°C while gently blowing a stream of air across the surface. When the volume was reduced to about 5 ml, aliquots were removed for radioassay and determination of glucose concentration.

To maintain the hydrolysates of liver and muscle glycogen in a concentrated form and in a known volume, they were diluted in 25 ml volumetric flasks. Two ml

aliquots from the liver hydrolysates were neutralized and diluted appropriately for glucose assay. Since the hydrolysates of muscle glycogen were much lower in glucose initially, a second dilution was not necessary, and glucose was determined on 0.15 ml portions from the 25 ml volumetric flask.

The remaining 23 ml of each hepatic glycogen hydrolysate was passed through a 1.2 X 30 cm column of Amberlite MB-3 ion exchange resin to remove charged substances. The columns were rinsed with water at a drip rate of 15 drops per minute. To obtain a solution which contained the greatest concentration of glucose, four 25 ml fractions were collected from every column, and each fraction was assayed for glucose. The second fraction almost always contained the greatest concentration and was generally used for radioassay. If all fractions were found to have glucose concentrations that were too low to give significant counts above background, all four fractions were combined and concentrated by evaporation of water.

For muscle glycogen, a portion of each hydrolysate was added to a centrifuge tube containing Amberlite MB-3 ion exchange resin. An excess of resin was used, as judged by the failure of the indicator in the anion exchanger to change color. The solution and resin were swirled vigorously until thoroughly mixed, then centrifuged at 2000 rpm for 15 minutes to pack the resin.

Aliquots were used for the determination of glucose and for radioassay.

All glucose solutions were assayed for radioactivity by placing a 0.5 ml aliquot in 10.0 ml of diotol scintillator solution, as described by Buhler (9). Counting was performed in a Tri-Carb model 3000 Liquid Scintillation Spectrometer (Packard Instrument Co.) at $0^{o}C$ with an 8.5% gain and a window calibration of 70-1000. The efficiency of this method with this equipment was determined to be 78.2% by placing an aliquot of a diluted toluene-C^{14} Standard (Packard Instrument Co.) with 0.5 ml of water in 10.0 ml of the scintillator solution. An aliquot of the same standard solution was added to each sample after it was counted and then recounted to insure that the glucose solutions had no quenching effect. The calculated specific activity of the glucose from each source is expressed as disintegrations per minute per uMole of glucose (dpm/uM).

For the normal distribution of "t" values, the table in the appendix of McNemar's Psychological Statistics (New York: John Wiley & Sons, Inc., 1962) was accepted for comparison with the values calculated from the data, and statistical interpretations of the data were made on the basis of small sample theory using this comparison.

R E S U L T S

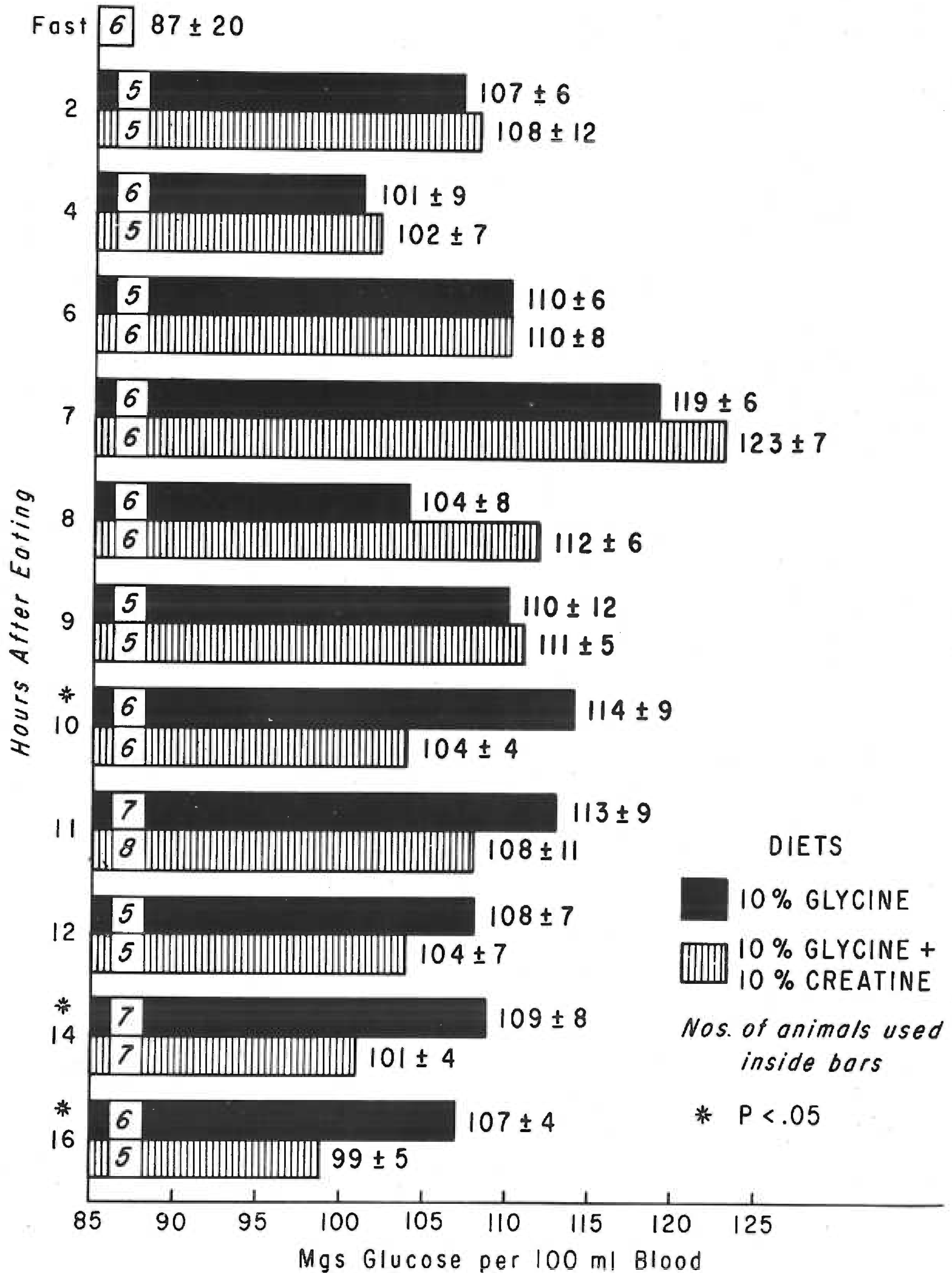
Nonisotopic Studies:

Figures #1-#3 express graphically the levels of blood glucose, liver glycogen, and muscle glycogen obtained from 48-hour fasted, male rats, before and at various times from 2-16 hours after feeding the experimental diets. All three graphs are labeled similarly with the mean for each group placed at the end of each bar and followed by the value of one standard deviation of the mean. The number of animals contributing values to each group is inside each bar for that group. The 10% glycine diet will be referred to as the glycine diet. The diet containing 200 mg of creatine added to 2.0 g of 10% glycine diet is labeled as 10% glycine plus 10% creatine ration and is referred to as the creatine diet in the discussion.

Blood glucose. Figure #1 demonstrates the values for blood glucose in mg glucose per 100 ml blood. No differences in the level of blood glucose between glycine-fed and creatine-fed animals were apparant for the first 9 hours after eating the 2 g of diet, but it is interesting to note that the values for the creatine-fed rats were equal to or greater than those of the glycine-fed animals at every time interval measured during this period. Starting at 10 hours after eating the diets, the reverse was true; the blood glucose

Figure #1

Blood glucose levels, mg glucose/100 ml blood, obtained from 48-hour fasted male rats before and 2-16 hours after eating 2.0 g 10% glycine diet or 2.0 g 10% glycine diet plus 200 mg creatine during a single 30-minute feeding period. All times, in hours, are measured from end of feeding period. At the end of each bar is the mean value for the number of observations inside the bar plus or minus one standard deviation of the mean.


BLOOD GLUCOSE LEVELS IN 48 HOUR FASTED RATS
AFTER 2.0 GRAMS OF DIET
Fast
6
87 ± 20
2
5
107 ± 6
5
108 ± 12
4
6
101 ± 9
5
102 ± 7
6
5
110 ± 6
6
110 ± 8
7
6
119 ± 6
6
123 ± 7
8
6
104 ± 8
6
112 ± 6
9
5
110 ± 12
5
111 ± 5
*
10
6
114 ± 9
6
104 ± 4
11
7
113 ± 9
8
108 ± 11
12
5
108 ± 7
5
104 ± 7
*
14
7
109 ± 8
7
101 ± 4
*
16
6
107 ± 4
5
99 ± 5
Hours After Eating
85
90
95
100
105
110
115
120
125
Mgs Glucose per 100 ml Blood
DIETS
10 % GLYCINE
10 % GLYCINE +
10 % CREATINE
Nos. of animals used
inside bars
* P < .05

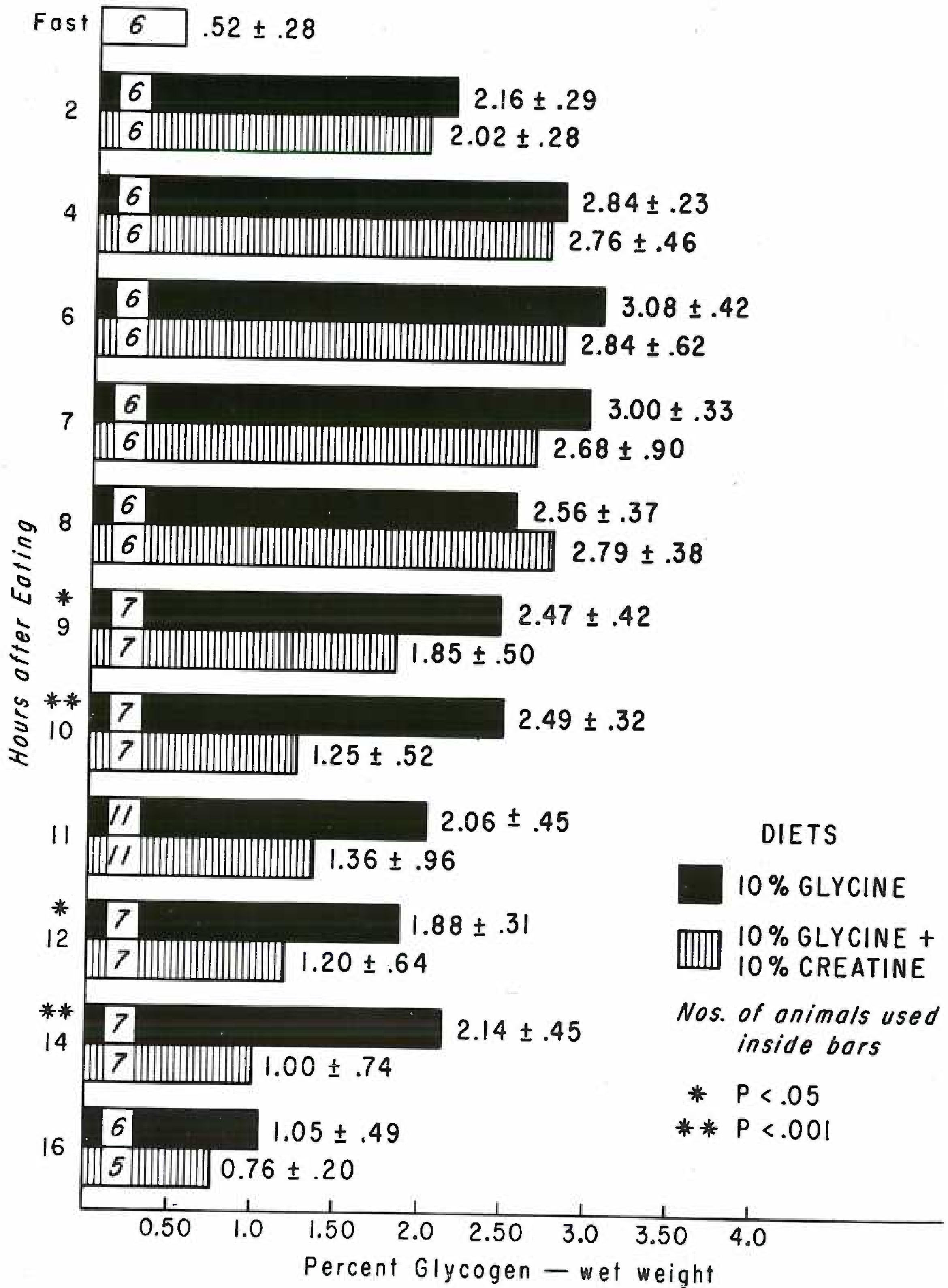
levels in the glycine-fed animals exceeded those of the creatine-fed animals at every point from 10 to 16 hours. The values at 10, 14, and 16 hours after eating showed differences between the groups that are statistically significant.

Liver glycogen. Figure #2 illustrates the levels of liver glycogen, expressed as per cent wet weight of liver. No significant differences were observed between the creatine-fed and glycine-fed animals during the first eight hours after eating, during which time the glycogen levels were increasing, plateauing, and beginning to decrease. During the period of decreasing liver glycogen levels from 9 to 16 hours following food intake, the glycine animals consistently exhibited greater levels of liver glycogen, although the points at 11 and 16 hours after eating were not statistically different.

The tendency for greater variability of the levels of liver glycogen was characteristic among the animals receiving creatine in their diet. At every time interval measured, except 2 and 16 hours after eating, the standard deviation for the group of creatine-fed animals was greater than the companion value for the glycine animals. The variability was greatest from 9 to 16 hours after eating when the creatine animals evidenced rapidly decreasing liver glycogen levels. During this period the largest standard deviation of any glycine

Figure #2

Liver glycogen levels, percent wet weight, obtained from 48-hour fasted male rats before and 2-16 hours after eating 2.0 g 10% glycine diet or 2.0 g 10% glycine diet plus 200 mg creatine during a single 30-minute feeding period. All times, in hours, are measured from end of feeding period. At the end of each bar is the mean value for the number of observations inside the bar plus or minus one standard deviation of the mean.



LIVER GLYCOGEN LEVELS IN 48 HOUR FASTED RATS
AFTER 2.0 GRAMS OF DIET
Fast
6
.52 ± .28
2
2.16 ± .29
2.02 ± .28
4
2.84 ± .23
2.76 ± .46
6
3.08 ± .42
2.84 ± .62
7
3.00 ± .33
2.68 ± .90
8
2.56 ± .37
2.79 ± .38
* 9
7
2.47 ± .42
1.85 ± .50
** 10
2.49 ± .32
1.25 ± .52
11
2.06 ± .45
1.36 ± .96
* 12
1.88 ± .31
1.20 ± .64
** 14
2.14 ± .45
1.00 ± .74
16
5
1.05 ± .49
0.76 ± .20
Hours after Eating
0.50
1.0
1.50
2.0
2.50
3.0
3.50
4.0
Percent Glycogen — wet weight
DIETS
10% GLYCINE
10% GLYCINE + 10% CREATINE
Nos. of animals used inside bars
* P < .05
** P < .001

group failed to exceed the smallest standard deviation of any creatine group. At 11 hours the large standard deviation of the values for the creatine-fed animals precludes the apparent large decrease in glycogen level from constituting a significant difference.

The comparable levels of liver glycogen between the groups demonstrated a slightly different pattern from that found in the blood glucose of these animals (Figure #1). Although the blood glucose levels among the animals receiving dietary creatine tended to be equal to or slightly higher than those in the glycine-fed rats during the first 9 hours after eating, their liver glycogen levels were slightly lower during this same period, except for the 8-hour point. The first significant difference between groups was not apparent in the blood glucose levels until 10 hours after eating, but statistically significant differences were demonstrated in the liver glycogen by the 9th hour. After the 9-hour point, blood glucose and liver glycogen levels were lower at each time interval in the animals given the creatine diet compared to those fed the glycine ration alone. These decreases were concurrently statistically significant at 10 and 14 hours following consumption of the diets; however, the liver glycogen levels evidenced other significant differences at a greater number of points, and the differences were of greater magnitude statistically than those found in blood glucose.

Muscle glycogen. Figure #3 shows the levels of muscle glycogen, expressed as per cent wet weight. Of the three parameters observed during these experiments, muscle glycogen showed the least absolute and percentage increase immediately following eating. This was true for both groups of animals and reflects the relative immobility of this polysaccharide under the experimental conditions employed. Muscle glycogen levels were not constant, however, as figure #3 demonstrates, but were continually undergoing small vacillations during the course of the experiments.

The animals receiving dietary creatine showed their highest level of muscle glycogen at 6 hours after eating. At this same time interval, the glycine-fed animals exhibited one of the lowest levels of muscle glycogen for any time interval measured, and the difference between the groups was statistically significant. In spite of this difference, the pattern of comparative muscle glycogen levels between groups at the remaining time intervals was similar to that observed in the case of liver glycogen (Figure #2). From 9 to 16 hours after eating, the animals receiving creatine in their diet demonstrated decreased levels of muscle glycogen with statistically significant differences at 10, 14, and 16 hours.

The data presented indicate that there is little difference in the levels of blood glucose, liver glycogen,

and muscle glycogen during the first 9 hours after feeding. Beginning at 9 hours for tissue glycogens and 10 hours for blood glucose, the animals receiving the creatine diet exhibited decreased levels until as late as the 16th hour after food intake. The significance of these decreases varied statistically for each substance at each time interval observed. Concurrent statistically significant decreases in levels occurred in the creatine-fed rats at 10 and 14 hours after eating.

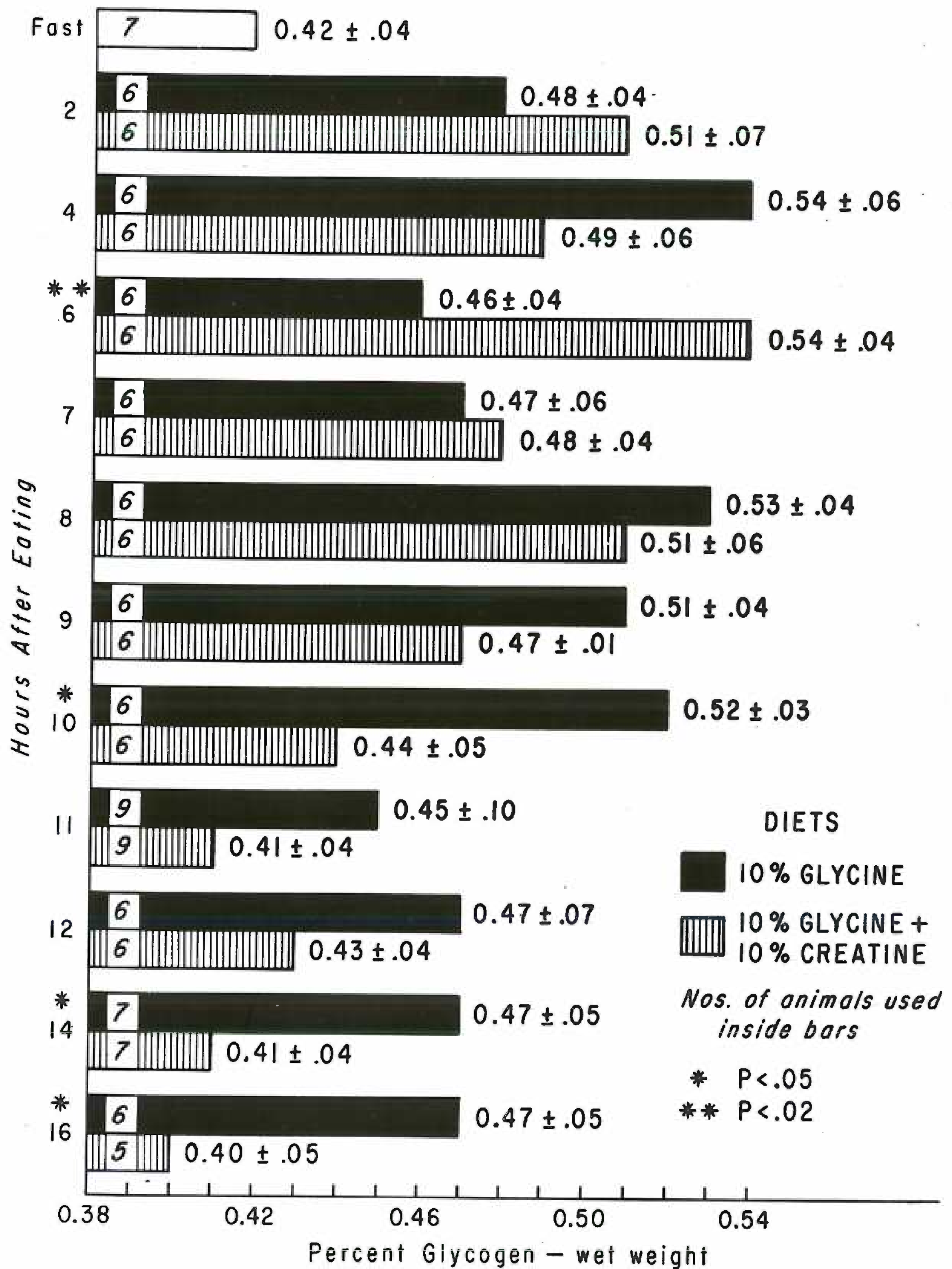
Preliminary Isotopic Studies:

Tables #1, 2, and 3 summarize the results obtained in 48-hour fasted rats after consuming each of the two diets. The animals were administered 11 uC of glucose-U-C^{14} at 5 hours after eating and sacrificed at hourly intervals thereafter up to 10 hours. These tables present values for levels of blood glucose, liver glycogen, and muscle glycogen, as well as the specific activity of the glucose from each source. The values for the level and the specific activity of each carbohydrate examined from glycine-fed and creatine-fed rats are aligned in columns under the number of hours after eating and the hours after receiving the isotope in order to facilitate interpretation. Each value is the mean value for the number of animals in the parenthesis for that group and each mean is followed by the value for one standard deviation of the mean. The specific

Figure #3

Muscle glycogen levels, percent wet weight, obtained from 48-hour fasted male rats before and 2-16 hours after eating 2.0 g 10% glycine diet or 2.0 g 10% glycine diet plus 200 mg creatine during a single 30-minute feeding period. At the end of each bar is the mean value for the number of observations inside the bar plus or minus one standard deviation of the mean.

MUSCLE GLYCOGEN LEVELS IN 48 HOUR FASTED RATS
AFTER 2.0 GRAMS OF DIET



activities are expressed as disintegrations per minute per uM of glucose.

Blood glucose. Table #1 demonstrates the values for blood glucose 6-10 hours after eating and 1-5 hours after the tracer injection. In these experiments, with only 2 or 3 animals at most points, no significant differences in levels of blood glucose were apparent between glycine-fed and creatine-fed animals. The specific activities of the two groups 1 hour after injecting the glucose-C^{14} were equivalent and decreased equally as logarithmic functions for the next 3 hours. Following the initial 3-hour period after the injection, the values for the glycine-fed animals continued to approximate the initial rate of logarithmic decrease, but the creatine-fed rats exhibited a greater rate of decrease in specific activity at 4 hours after the injection and a lesser rate of decrease following this point. As a result of losing two samples from the glycine-fed animals 9 hours after eating, there is only 1 value for specific activity at this point. No significant difference between the groups was found at this point with only 4 animals. With a single additional glycine-fed animal at the 10-hour interval, however, the lesser difference was significant. Thus it is probable that a statistically significant difference existed between the groups at 9 hours also. Furthermore, the value for specific activity

TABLE #1

LEVELS AND SPECIFIC ACTIVITY OF BLOOD GLUCOSE IN RATS

6-10 Hours After Eating Experimental Diets

Hours After Feeding 2.0 Grams of Diet:	6	7	8	9	10
Hours After Injecting 11 uC Glucose-C-14:	1	2	3	4	5
BLOOD GLUCOSE LEVELS (mg. glucose/100 ml. blood)					
10% Glycine Diet:	110±3 (3)	124±7 (4)	122±4 (3)	123±6 (3)	126±6 (2)
10% Glycine + 10% Creatine:	122±6 (3)	127±10 (3)	124±6 (2)	121±8 (3)	127±9 (3)
BLOOD GLUCOSE SPECIFIC ACTIVITY (dpm/uMole glucose)					
10% Glycine Diet:	13,780 ±1660 (3)	6,506 ±612 (4)	3,794 ±162 (3)	1,783 (1)	1,318* ±45 (2)
10% Glycine + 10% Creatine:	13,543 ±1120 (3)	5,645 ±770 (3)	3,778 ±344 (2)	778 ±170 (3)	712 ±45 (3)

Mean Values± Standard Deviation
(#)=Number of Animals
*= P <.01

obtained from the 1 glycine-fed animal 9 hours after feeding appeared lower than the specific activities observed in the glycine-fed animals at the other 4 intervals would indicate. If a graph were drawn of log blood glucose specific activity as a function of time, using the data in Table #1 for glycine-fed rats, 4 of the points would approximate a straight line function, but the value 9 hours after eating would fall below the line. Since it is also difficult to explain a true decrease in specific activity at this point followed by a subsequent rise to fit the graph again at 10 hours, it is assumed that the true value at 9 hours after eating was higher than the table indicates.

Liver glycogen. Table #2 gives the values for liver glycogen 6-10 hours following the ingestion of the experimental rations and 1-5 hours following the injection of glucose-C^{14}. Because of the small sample sizes and the large variations that occurred in the values between animals in these experiments, the apparent decreases in liver glycogen levels at 9 and 10 hours after eating the creatine-supplemented ration were not shown to be statistically significant. The pattern of liver glycogen decrease in creatine-fed animals was in fair agreement with the pattern found in the nonisotopic studies.

The pattern of glucose-C^{14} incorporation into liver glycogen in the 2 groups of animals during the 5 hour

TABLE #2

LEVELS AND SPECIFIC ACTIVITY OF LIVER GLYCOGEN IN RATS

6-10 Hours After Eating Experimental Diets

Hours After Feeding 2.0 Grams of Diet:	6	7	8	9	10
Hours After Injecting 11 uC Glucose-C-14:	1	2	3	4	5
LIVER GLYCOGEN LEVELS (per cent wet weight)					
10% Glycine Diet:	2.72±.35 (3)	3.18±.18 (4)	2.38±.51 (3)	2.33±.49 (3)	2.89±.15 (2)
10% Glycine + 10% Creatine:	2.83±.59 (3)	2.54±.65 (3)	2.34±.02 (2)	0.95±.76 (3)	1.81±.37 (3)
LIVER GLYCOGEN SPECIFIC ACTIVITY (dpm/uMole glucose)					
10% Glycine Diet:	111±16 (3)	193±23 (4)	106±98 (3)	80±29* (3)	138±23** (2)
10% Glycine + 10% Creatine:	68±44 (3)	98±90 (3)	184±18 (2)	12±9 (3)	27±7 (3)

Mean Values±Standard Deviation
(#)=Number of Animals
*= P<.05
**= P<.01

period following the isotope injection presents several contrasts. In the glycine-fed animals there was a fair correlation between hourly liver glycogen levels and the degree of glucose-C^{14} incorporation, although the latter varied to a greater extent. In the creatine-fed animals, such a pattern was not evident until the 4th and 5th hours after injection. In spite of equal or slightly decreasing levels of liver glycogen in creatine-fed animals for the first 3 hours after the injection, the specific activity of the glycogen in these animals continued to increase reaching a maximum value 1 hour later than was found in glycine-fed animals. Although there were apparent differences between the groups in the first 3 hours after injecting the tracer glucose, only the differences at 4 and 5 hours are statistically significant.

Muscle glycogen. Table #3 indicates the values for muscle glycogen 6-10 hours after consuming the experimental diets and 1-5 hours after the isotope injection. During these experiments no statistically significant differences were found between groups for either levels or specific activity of muscle glycogen. The failure of the levels found in these experiments to adhere to the previously observed pattern in nonisotopic studies, plus the large variations between rats at several of the points make interpretation of the data difficult.

TABLE #3

LEVELS AND SPECIFIC ACTIVITY OF MUSCLE GLYCOGEN IN RATS

6-10 Hours After Eating Experimental Diets

Hours After Feeding 2.0 Grams of Diet:	6	7	8	9	10
Hours After Injecting 11 uC Glucose-C-14:	1	2	3	4	5
MUSCLE GLYCOGEN LEVELS (per cent wet weight)					
10% Glycine Diet:	0.53±.02 (3)	0.60±.01 (3)	0.52±.04 (3)	0.57±.01 (2)	0.50±.04 (2)
10% Glycine + 10% Creatine:	0.52±.03 (3)	0.55±.02 (3)	0.54±.01 (2)	0.52±.06 (3)	0.58±.04 (3)
MUSCLE GLYCOGEN SPECIFIC ACTIVITY (dpm/uMole glucose)					
10% Glycine Diet:	344±83 (3)	167±18 (3)	412±293 (3)	537±261 (3)	187±32 (2)
10% Glycine + 10% Creatine:	157±66 (3)	191±45 (3)	404±166 (2)	120±75 (3)	191±47 (3)

Mean Values± Standard Deviation

(#)=Number of Animals

No Significant Differences (P=.05)

The specific activities of muscle glycogen for both groups were greater at almost every time interval than the values for liver glycogen in the same animals. The specific activity pattern in the creatine-fed animals tended to duplicate that found in the liver glycogen. The point of maximum incorporation occurred at 8 hours after eating, followed by a marked decrease in specific activity at 9 hours. The glycine-fed animals did not demonstrate an equivalent decrease until the 10th hour after eating. Although the muscle glycogen levels for both groups were identical one hour after injecting the tracer glucose, the glycine-fed animals attained specific activities of greater than twice the values observed for creatine-fed animals during the same interval.

The data presented show that in these experiments no statistically significant differences were detected in the levels of carbohydrate that were studied in glycine-fed and creatine-fed rats, partly due to the small number of animals used at each point in the experiments. Blood glucose, liver glycogen, and muscle glycogen specific activity evidenced a greater decrease in the creatine-fed animals 9 hours after eating the experimental diets than did the corresponding specific activities in the glycine-fed animals. Only the difference in the specific activities of liver glycogen is of

statistical significance between the groups at this point. At 10 hours after eating, the creatine-fed animals continued to show much lower specific activities in liver glycogen and blood glucose compared to the values observed in the glycine-fed animals. These differences were statistically significant. No significant differences were found between the groups in muscle glycogen.

D I S C U S S I O N

Several workers have demonstrated increased glycogen formation in rats (2, 10, 22) and mice (24) following the oral administration of glycine. Todd et al. (35) observed that rats prefed a diet containing 10% glycine for 24 hours prior to a cold swim stress showed a marked increase in their liver glycogen stores following a recovery period after the stress. When animals were fed this diet containing an additional 1% creatine, this capacity was lost (36). Furthermore, the animals receiving 1% dietary creatine had prestress liver glycogen levels that were less than half those in rats fed the diet without added creatine. Decreased blood glucose values were also noted in the creatine-fed animals after the recovery period. No differences were found by these workers in the blood glucose values before stress, or in muscle glycogen values at either point in the experiment when the diet included creatine.

The studies presented in this thesis differ in design from those conducted by Todd et al. (36). A fast, to reduce carbohydrate stores preceded feeding the two rations, and the total amount of food intake was only 2 g compared to the 15 to 20 g which Todd et al. fed in a 24-hour period. The amounts of creatine ingested per rat were comparable in the 2 studies, about 200 mg. The results from the present studies substantiate and extend the previously mentioned

decrease in rat liver glycogen following the ingestion of dietary creatine (Figure #2). When food intake and timing were closely controlled, dietary creatine effects occurred from 9 to 16 hours after feeding. With the conditions employed in the present studies, it is apparent that the levels of blood glucose (Figure #1) and muscle glycogen (Figure #3) were also depressed from 10 to 16 hours after eating the creatine-supplemented diet. The timing for the appearance of these decreases after creatine ingestion is in good agreement with previous studies.

Liver glycogen and muscle glycogen levels serve as an indicator of the level of body carbohydrate stores, and blood glucose values act as an index of the level of the extracellular glucose pool. It follows then that the major effect of dietary creatine in these studies with rats was to produce a reduction in amounts of both carbohydrate stores and extracellular glucose after 9 hours following intake of the rations, since all three indices remained consistently lower in the creatine-fed animals during this period. The effect must also be somewhat of a cyclic nature since even though the creatine-fed animals exhibited lower carbohydrate levels than the glycine-fed rats throughout the 10- to 16-hour period after feeding, only the decreases at 10 and 14 hours are concurrently significant in all three parameters.

Also, the decreases at 11 hours after feeding are not different statistically in any of the three substances measured. The fact that the points at 9, 12, and 16 hours after feeding show differences between the groups for levels of 1 or 2 indices, but not the remaining 1 (2) is most likely a function of animal variability, differential organ response, and the arbitrarily chosen time intervals for measurement. It should be pointed out that the statistically significant increase of the level of muscle glycogen that occurred 6 hours after feeding creatine is apparently an artifact since it does not fit the pattern for the values of the other 2 indices and is quite removed from any of the other noted changes.

A comparison of these studies with the findings of Todd and coworkers (36) may be limited in value since the significance of adaptive changes induced in these animals with 48 hours of fasting and the influence of these changes on the effects of dietary creatine are unknown. The fast was used to encourage the animals to eat the experimental diets readily and to reduce the total carbohydrate level of the animals so that the effects of limited food intake might be observed more precisely.

The mechanism by which dietary creatine produced a decrease in both the carbohydrate stores and the extracellular glucose pool is not clear, but several

possibilities exist. As with the alteration of level of any metabolic pool, the explanation must be reached by considering those factors that influence the rate of inflow of metabolites into the pool and those that affect the rate of outflow from the pool.

Considering factors which decrease glucose inflow into the carbohydrate pool, an irritant action of creatine on the small bowel could decrease the transit time of foods through this portion of the gut. These animals might absorb less of the dietary foodstuffs and show carbohydrate shortages later. Several observations do not support this possibility. During the experiments dietary creatine was never observed to produce diarrhea, nor were differences between the glycine-fed or the creatine-fed animals observed as far as behavior which might indicate distress. If the creatine animals had absorbed less food due to bowel irritation, the decreased glucose absorption should have been reflected in the blood glucose concentrations early in the experiment. After eating, however, the creatine animals exhibited blood glucose levels that were equal to or greater than the glycine-fed animals at every interval measured for the first 9 hours (Figure #1). Intestinal irritation is apparently not involved.

A more specific mechanism involving the intestinal mucosa is suggested by the similarity of structure of

creatine and glycine. There may be competition for the same transport mechanism across the gut wall, in which case glycine absorption could be decreased, and these animals would lose a part of their diet. Competition for a common transport mechanism for glycine and creatine has been shown to exist in the kidney by Pitts (27). Several observations by other investigators make this possibility seem unlikely. Fitch (14) failed to notice any reduction in the urinary creatine to creatinine ratio when rats on a 1% creatine diet were fed this diet containing 6% glycine. Such a reduction would be expected if glycine were antagonizing the intestinal absorption of creatine to a significant extent, unless the affinity of the transport mechanism was much greater for creatine, which seems unlikely. Similarly, Todd et al. (36) observed no differences in daily food consumption or weight gain over a 7-day period, between animals receiving a 10% glycine diet and animals eating a 10% glycine plus 1% creatine diet.

If the decreased carbohydrate levels of creatine-fed rats are to be explained on the basis of decreased inflow into the glucose pool, it is necessary to examine other routes of inflow. Many investigators have pointed out that the amount of glycogen formation from oral glycine is greater than can be explained by the direct carbon to carbon conversion of glycine to glycogen.

There is a good deal of evidence to suggest that exogenous glycine increases gluconeogenesis, but the site of action and the mechanism are not clearly established (1, 24, 35). Todd et al. (36) have offered an explanation for the decreased liver glycogen levels observed in animals fed 10% glycine plus 1% creatine, based on the inhibition of the gluconeogenesis produced by glycine. Such an explanation is attractive with regard to the timing of changes in the present studies. Early investigators have noted that the increased liver glycogen formation, and presumable increased gluconeogenesis, began 6-8 hours after animals ingested glycine, and became maximal at 14 hours (1, 19, 22). This time sequence agrees well with the times in the present studies at which the depression of carbohydrate levels occurred in the animals receiving creatine and glycine. The point at which the effect of glycine was found to be maximal by Hess et al. (19) and Barnet et al. (1) was 14 hours after eating. This corresponds closely to a time in the present studies when large differences were noted between glycine-fed and creatine-fed animals in all 3 carbohydrate parameters observed. A reduction in the production of glucose from other substances would explain these decreases in creatine-fed rats.

If the inhibition of glycine-induced gluconeogenesis were to explain the reduced carbohydrate levels in animals receiving creatine, then it could be postulated that the

similarity of the structures for glycine and creatine permit competition at some, as yet, unidentified site. The finding by Todd's group (36) that other glycine and creatine-related compounds would produce the same effect as creatine when substituted in the diet argues strongly for structural antagonism as a mode of action, rather than some nonspecific effect of added creatine in the diet. These investigators also noted, however, that the decrease in prestress levels of liver glycogen were not glycine dependent since animals fed a similar ration with 1% creatine, and no added glycine showed similarly decreased glycogen levels. This observation is strong evidence against a competitive inhibition of increased gluconeogenesis since glycine was not present to stimulate it.

Pitts (27) demonstrated that glycine and creatine compete for a common reabsorptive mechanism in the kidney tubule of the dog. Thus, the possibility exists that glycine is lost in the urine when creatine is fed. In spite of the fact that this investigator observed large increases in the renal excretion of endogenous or exogenous creatine when glycine infusions were begun, the reverse did not hold true. Even though high levels of plasma creatine were obtained, no glycine was lost. He suggested that there is a greater affinity of the common reabsorptive mechanism for glycine than for creatine.

Another major consideration for a mechanism of regulating carbohydrate stores and the level of the extracellular glucose pool in creatine-fed animals involves the pathways which remove glucose. An increased rate of removal of glucose from the extracellular glucose pool by any process would decrease the level of glucose in the pool and require breakdown of carbohydrate stores to replenish it. A constant glucosuria would be one such process. Unfortunately, the urine during these experiments was not checked for glucose; however, Todd et al. (36) found no glucose in the urine in their studies when a 1% creatine diet was fed. Pitts (27) found that the reabsorptive mechanisms in the dog kidney for glycine and creatine were independent of those concerned with glucose reabsorption. Increased plasma levels of glycine or creatine failed to produce glucosuria. In the light of these observations, the possibility of lowered carbohydrate stores through renal losses appears to be remote.

It is possible that glucose is removed from the extracellular pool and converted to fat or protein as a result of a stimulant action of creatine on these pathways. Since little is known about fat metabolism with regard to creatine feeding, this possibility cannot be excluded. With regard to stimulated protein synthesis, Hunter (21) has observed a close negative correlation

between levels of liver glycogen and amounts of hepatic mitochondrial nitrogen in rats prefed 10% glycine plus 1% creatine following a cold swim stress. This observation suggested that creatine may have some directive influence on protein metabolism. The explanation for stimulated protein synthesis seems less likely when it is considered that the effect occurs at a time when amino acids are more likely to be broken down to produce glucose.

Of the remaining possibilities such as diet induced stress or increased metabolic rate engendered by the additional creatine, little can be said for lack of information, but one possibility deserves further consideration. The fact that blood glucose, liver glycogen, and muscle glycogen levels decreased significantly and simultaneously at 10 hours after feeding creatine might indicate that the confines of the extracellular glucose pool have suddenly increased. This situation would be the case if creatine were to alter membrane permeability or stimulate peripheral uptake of glucose to such a degree that a previously inaccessible compartment were suddenly made available to extracellular glucose. Such an effect might be indirect and mediated through increased insulin activity. Since the creatine ordinarily synthesized by the liver is known to be stored as creatine phosphate in muscle tissue, it is

possible that the increased plasma concentration of creatine exerts some direct effect on muscle cell. Ord et al. (25) have shown that fluctuations of blood glucose concentrations affect the movement of creatine in and out of muscle cells. It is possible that the reverse is true. Increased creatine may require additional glucose uptake for some process such as oxidative phosphorylation. In such a case no increase, but even a decrease in muscle glycogen, as was observed in the present studies, might occur (Figure #3). Benedict et al. (3) have shown that small amounts of dietary creatine are converted to creatinine, implying that they have passed through creatine phosphate, a pathway established by the work of Borsook et al. (8). Fiske et al. (13) found no increase in creatine phosphate after injecting creatine, however. Van Pilsum's studies (41) showing that 83% of injected creatine was excreted in the urine in 48 hours, do not decrease the possibility that large amounts of creatine were bound temporarily by some complex intracellularly.

Preliminary Isotopic Studies:

To facilitate understanding, that space in the rat containing glucose which is in free equilibrium with blood glucose is defined as the extracellular glucose pool in this discussion. The equal levels and specific

activities of blood glucose for glycine-fed and creatine-fed animals 1 hour after injecting the glucose-C^{14} indicate that the extracellular glucose pools were nearly equivalent in glucose content for both groups (Table #1). The equal hourly levels of blood glucose and equal rates of decrease of blood glucose specific activity during the period from 6 to 8 hours after feeding the diets indicate that no differences existed between the groups of animals for rates of influx and outflux of glucose from the extracellular pool. Beginning at 9 hours and established statistically by 10 hours after eating, the specific activity of glucose for the creatine-fed rats decreased at a greater rate to produce a value half that found in glycine-fed rats. During this same period the blood glucose levels remained constant between the groups in these studies, although the glucose levels were shown to decrease in the creatine-fed rats in the nonisotope studies at 10 hours after eating (Figure #1). This relative decrease in specific activity represents a dilution of the extracellular glucose pool by glucose of lower specific activity and indicates that some pathway contributing glucose to the pool must have accelerated. If this dilution of specific activity to half that observed in the glycine-fed animals at an equivalent time had occurred predominantly as a result of increased glucose input to the extracellular pool, then the

failure of the blood glucose level to rise must mean that the ouflux of glucose from the extracellular glucose pool had increased by approximately two fold. Only if dilution had occurred from free equilibration of the extracellular glucose pool with some previously inaccessible portion of the total body glucose pool of equal size and glucose concentration, could the observed blood glucose dilution have occurred with no net loss of glucose from the extracellular pool. In such a case there would be no increased inflow of glucose from any source necessary to maintain a constant level of blood glucose. Reasoning will be presented to show that glucose inflow into the extracellular pool is increased and the source of dilution must, at least in part, be liver glycogen.

Stetten and Stetten have shown that liver glycogen is an inhomogenous substance (31-34). In tracer studies of less than 12 hours duration in rats, the incorporated radioactivity was restricted to the outer tiers of the glycogen molecules, and the smaller molecules incorporated activity to a greater extent. These findings may have a bearing on the interpretation of data obtained in the present study.

A comparison of rates of glucose-C^{14} incorporation into liver glycogen depends upon the assumption that the precursor glucose is equally labeled for both groups.

Cahill et al. (11) have shown that blood glucose is in free equilibrium with liver cell glucose. Thus, the findings of equal, hourly, blood glucose specific activities in both glycine-fed and creatine-fed groups satisfies this assumption for the first 3 hours after injection of the tracer glucose, so long as it is also assumed that the conditions of the experiment did not alter liver cell membrane permeability in any way.

The presence of approximately equal levels of liver glycogen in both groups of rats from 6 to 8 hours after eating, indicates that no alterations in net synthesis resulting from glycogen synthesis and degradation existed between the groups (Table #2). The lower specific activities of liver glycogen in the creatine-fed animals 1 hour after injection of the tracer glucose and the hour delay in achieving a maximum level of specific activity compared to the glycine-fed animals suggest that the rate of glycogen synthesis in the creatine-fed animals was less than that observed for the glycine-fed animals. Since no statistically significant differences were found during this period, the evidence is not conclusive.

At 9 and 10 hours after eating, the decreased liver glycogen levels in creatine-fed rats is evidence that the rate of glycogen degradation was predominating over the rate of glycogen synthesis in these animals. The resultant removal of previously incorporated tracer

glucose from the outer layers of the glycogen molecules is reflected in the low specific activity of the remaining liver glycogen in these animals.

Comparing these findings with the relative changes observed in blood glucose specific activity at the same time intervals in the creatine-fed animals, it can be concluded that the rate of glycogen degradation must have undergone an absolute increase. Since the specific activity of liver glycogen at 8 hours after eating was small in comparison to blood glucose specific activity, the breakdown of liver glycogen could explain the dilution of blood glucose specific activity, in the creatine-fed animals to amounts that were only half of those observed in the glycine-fed animals 9 and 10 hours after eating. A decrease or complete block in the rate of synthesis of liver glycogen in the presence of a constant rate of breakdown in these animals would explain the reduced level of glycogen, but does not account for the large dilution of blood glucose specific activity that occurred. Since some constant rate of dilution of blood glucose specific activity must have occurred during the first 3 hours after the tracer injection by virtue of the fact that any liver glycogen breakdown returned glucose of low specific activity to the blood, blocking the pathway of glycogen synthesis would not explain the additional dilution which occurred at 9 to 10 hours after eating in the animals receiving creatine.

Since an absolute increase in the rate of glycogen breakdown occurred in the rats receiving dietary creatine at 9-10 hours after eating, yet the blood glucose levels did not rise in these animals, it follows that there must have been an increased rate of glucose removal from the extracellular pool to some unknown site. Depending on the magnitude of increased free equilibration, if any increase, between the extracellular glucose pool and the total body glucose pool, the rate of glucose removal must have been increased by some factor up to twice the original rate. The absolute increase in the rate of liver glycogen degradation is probably secondary to the increased removal of glucose from the extracellular pool.

In muscle glycogen (table #5), the results observed for levels of muscle glycogen in the tracer studies do not agree closely with the levels found in the nonisotope studies, but the results do not detract from the explanation of the changes observed in blood glucose and liver glycogen in these experiments. Since muscle glycogen cannot be broken down to release glucose directly into the blood, a dilution from this carbohydrate store would have to be mediated through the liver and subject to the direction of hepatic metabolic flow in any event. The most important finding concerning muscle glycogen in these studies is that changes observed in this carbohydrate store cannot account for

the increased loss of glucose from the extracellular pool 9 and 10 hours after eating in the animals receiving creatine. If this increased outflux of glucose in these animals is due to increased uptake by muscle cells, the glucose evidently was not converted to muscle glycogen, to any great extent.

One of the possible explanations mentioned earlier for the effect of dietary creatine was the inhibition of gluconeogenesis and the findings in these studies with regard to this consideration should be clarified. The evidence presented points to a reduction in carbohydrate levels as a result of increased outflux from the extracellular glucose pool, rather than a decreased inflow into this pool. It is possible that this mechanism may be operating in addition to those discussed, but the changes incurred by the increased rate of glycogen degradation are so dramatic as to obliterate any evidence of this possibility.

The changes that were found in these studies have been continually discussed as the effects of creatine feeding. It is possible that a contaminant of creatine is responsible. In rats fed alacreatine Fitch and Dinning (15) have recently noted changes which were no longer present when the compound was highly purified and refed. This point is included for consideration, but it is doubtful that such a factor is active in these studies

since none of the toxic manifestations that these workers reported were observed in the present studies.

S U M M A R Y

It has been observed in studies by other investigators that rats prefed diets containing 10% glycine had an increased ability to redeposit liver glycogen after a recovery period following stress. When the same diet containing 1% creatine was fed, the animals not only lost this ability, but also had lower blood glucose levels. In addition, the liver glycogen levels in creatine-fed rats prior to stress were less than half those found in animals fed the diet without creatine.

In this thesis studies are presented that have been conducted in rats with the conditions of the experiment altered to reduce carbohydrate stores prior to feeding the diets. The time and quantity of food intake were standardized by restricting the meal to 2 g of diet in a 30-minute period. With this design certain aspects of carbohydrate metabolism could be observed closely in an anabolic and catabolic phase during the following 16 hours.

No significant differences in blood glucose or tissue glycogen levels between glycine-fed and creatine-fed rats were found during the first 8 hours after eating. After 8 hours for hepatic and muscle glycogen and after 9 hours for blood glucose, decreased levels were observed in the creatine-fed rats compared to the levels in animals receiving no creatine. These decreases

from 9 to 16 hours after eating were statistically significant at various times for each substance, but all exhibited statistically significant decreases at 10 and 14 hours after feeding.

Preliminary studies with glucose-C^{14} injected intraperitoneally 5 hours after the experimental rations were eaten indicated that the reduction of liver glycogen levels in creatine-fed rats was a result of an absolute in crease in the rate of liver glycogen degradation at 9 and 10 hours after eating. This increased rate of liver glycogen breakdown was probably secondary to the accelerated rate of removal of glucose from the extra-cellular glucose pool which was observed to occur simultaneously. The site of increased glucose outflow from the extracellular glucose pool was not identified. The possibility that increased amounts of glucose were shunted into fatty acid synthesis or taken up at some peripheral site as a result of the dietary creatine is discussed. With the limited data available, no conclusive explanation was reached.

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